

Spring 2019

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Recommended Citation

Young, William, "Exosomes and Their Complex Role in Pathogenesis" (2019). *Creative Components*. 287.
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BMS 599

Dr. Michael Kimber

Exosomes and Their Complex Role in Pathogenesis

Introduction

The intriguing nature of exosomes dates to the year 1983 when they were first reported (Harding et al., 1983). It was at this moment that a breakthrough had occurred, and an entirely new field of microbiology was opened. Our understanding of exosomes has gone from considering them merely waste buckets to recognizing them as key components of cellular communication and even therapeutic agents. Thousands of papers have been published regarding exosomes, a journal was created that bore the name, *Journal of Extracellular Vesicles*, and various societies were founded solely for exosomes. While it is widely accepted that exosomes are vital players in many significant functions of normal cells, along with diseased cells; the contribution that exosomes have on cellular communication has not always been clear and seems to take on an ever-evolving role that is hard to quantify or define. This role may be expanded in the near future to therapeutic treatment for a wide array of diseases. To fully appreciate what is currently understood on exosomes, and what their future holds, a background on exosomes is required.

Biogenesis

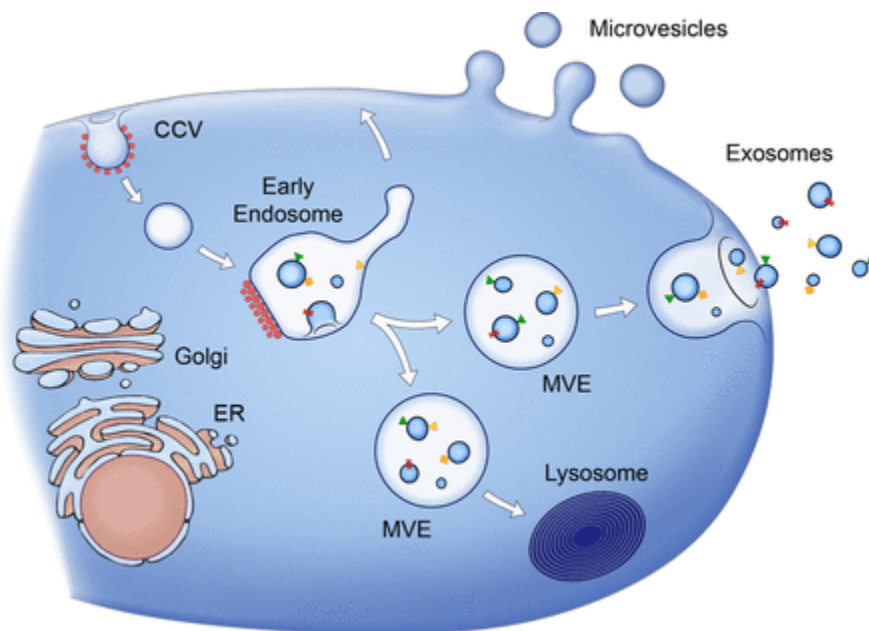
Exosomes arise from late endosomes during the endosomal pathway. These late endosomes have predominately two fates, fuse with lysosomes where the contents of the vesicle

can be degraded, or to be shuttled to the plasma membrane and be released as exosomes. These multivesicular endosomes that are not fused with lysosomes begin the biogenesis of exosomes. This process is the first step of exosomal secretion and involves many different proteins. Portions of the late endosome undergo inward budding to form ILV's or intraluminal vesicles. This process is carried out by the ESCRT complexes. ESCRT-0 is comprised of the essential protein Hrs (Hessvik et al., 2017). This complex is responsible for identifying and sequestering ubiquitinated proteins. ESCRT-I is comprised of the essential proteins TSG101 and STAM1 (Hessvik et al., 2017). ESCRT-I is primarily responsible for the budding and sorting of cargo in the vesicles. Several studies have been done to shown that a lack of ESCRT-0 and ESCRT-I complexes greatly reduces production of exosomes. ESCRT-I and ESCRT-II recruit ESCRT-III. The ESCRT-III complex and associated proteins CHMP4C, VPS4B, and VTA1 have been shown to play a role in vesicle scission and potentially inhibit the secretion of exosomes (Hessvik et al., 2017). Another protein associated with the ESCRT-III complex, ALIX, changed the protein composition of the exosome, signifying that it plays a role in the cargo loading of the exosomes and doesn't inhibit the secretion like the other proteins associated with the ESCRT-III complex (Hessvik et al., 2017). Another group of proteins responsible for the formation and secretion of exosomes are syndecans, which are membrane proteins. These proteins have been shown to bind ALIX and their binding seems to impact the formation of intraluminal vesicles.

Upon arrival at the plasma membrane, the multivesicular bodies are fused with the membrane via Rab GTPases. These Rab proteins are essential to the fusion and ultimately secretion of the exosomes. Rab11 mutants show inhibition of exosomal release in *drosophila* but do not inhibit exosome release in HeLa cells (Hessvik et al., 2017). However, a knockdown of the Rab35 protein did show a reduce in the exosome release of the proteolipid protein in

oligodendroglial cell line Oli-neu but not in *drosophila* (Hessvik et al., 2017). This protein is thought to help coordinate the docking of the multivesicular bodies to the plasma membrane. It is these ESCRT complexes and Rab proteins that will be touched on later due to their role during viral infections.

Lipids also greatly impact the formation and transportation of these vesicles. Ceramide, a waxy lipid molecule, seems to be of great importance to the exosomal release. When sphingomyelinase 2, which is an enzyme that produces ceramide from sphingomyelin, was inhibited, there was a decrease of exosomal secretion of the proteolipid protein (Hessvik et al., 2017). Another enzyme that plays a role is phospholipase D2 which is an effector of ADP ribosylation factor 6 which regulates ILV formation.



Raposo, G. & Stoorvogel, W. et al., 2013

Figure 1: Shows a clear distinction between exosomes and other types of extra cellular vesicles. As can be seen, the microvesicles bud directly from the plasma membrane whereas exosomes bud into multivesicular endosomes which then fuse with the plasma membrane and then exosomes are released.

Exosomal markers

Exosomes are small, 30-150 nm, vesicles that are released from late endosomes. This is in contrast with many other types of Extracellular Vesicles, EV's, that bud directly from the plasma membrane and are released into the extra cellular environment. To distinguish these vesicles from each other, markers are generally used. Exosomes usually contain certain cellular contents that can act as markers in identifying exosomes. In this sense, exosomes have become the 'fingerprints' of individual cells and can help to identify what type of cell an exosome came from as well as the health of the cell. Markers change due to viral vs bacterial infections and also differ when a cell has become cancerous. These usual markers include mRNA, miRNA, proteins and lipids. All of which differ by cell type and are used for cellular communication. In a study done by Dr. Joanna Kowal, western blots were done on several different sizes pellets to gauge the different proteins in exosomes versus differing EVs. The most common proteins found were CD9, CD63, and CD81 (Kowal et al., 2016). The proteins Alix, HSP70, and HSP90 are also very often found in most exosomes and can be used as markers (Kowal et al., 2016). These proteins are vital for studies that rely on and require exosomes as their primary focus of study. In many of the reports discussed, it is these markers that were keyed on and once identified, the vesicles could then be defined as exosomes.

Cancerous Exosomes

The role of exosomes in cancer development has garnered more scrutiny in the last decade as more information shows their vital role in cellular communication. There are several ways that exosomes that are derived from cancerous cells can contribute to the progression of cancer.

The first way that it is able to do this is by furthering the oncogenic nature of already existing tumor cells. These cancerous cells can horizontally transfer mutant proteins such as EGFRvIII (Epidermal growth Factor Receptor Variant 3) via exosomes (Salomon et al., 2015). This exchange information results in an increase of survival genes and a decrease of cell cycle inhibitors (Salomon et al., 2015).

The second major way that cancerous exosomes promote growth of the malignant tumor is by facilitating a favorable environment for the tumor. Tumor cells release exosomes that promote angiogenesis (Salomon et al., 2015).. It is this angiogenesis that allows the cancer to remain nourished and continue to grow. Myofibroblasts are also a huge part of the growth of the tumor, because they are the originator of many matrix remodeling proteins. These cancerous exosomes are released and function to support myofibroblasts thus playing a major part in aiding matrix remodeling proteins. Mu et al. shows that exosomes can degrade the extra cellular matrix by integrated proteases. This releases growth factors and cytokines within the extra cellular matrix producing further anti apoptotic effects while increasing proliferation of invasive cancer cells. Another way that a favorable environment is produced was demonstrated in a paper by Dr. Hector Peinado. It stated that melanoma cancer cells educate bone marrow cells in order to promote a favorable microenvironment for the primary tumor. They do this by using the MET receptor, elevating its expression during metastasis. The MET receptor tyrosine kinase is a receptor that promotes mitogenic cellular responses. In this way, the exosomes are essential in promoting a protumor environment.

There are many important correlations between exosomes and tumors that are useful for both understanding how exosomes help to spread cancer and potentially provide therapy. First, the amount of exosomes present in circulation has a direct correlation with the size of the tumor

(Fleming, A. *et al*). This could potentially be a novel way to determine the extent to which the cancer has spread and if markers can be identified, which tissues are compromised. Second, there is evidence that exosomes play a role in the direct migration of the tumor. In a study by Hood et al., 2011, cancer cells prepare distal tissues for colonization through exosomes. In the study it was shown that sentinel lymph nodes preferentially take up melanoma exosomes. Free melanoma cells are then recruited to the lymph nodes that have the melanoma exosomes and can subsequently be spread to distal tissue. This whole process requires the involvement of genes that control recruitment, angiogenesis, and extra cellular matrix modification.

Melanoma Signatures

One of the more well documented cases of cancerous exosomes was done by Dr. Deyi Xiao in the paper “Identifying mRNA, MicroRNA and Protein Profiles of Melanoma Exosomes” which shows the profiles of the genes, RNA and protein contents of exosomes derived from melanoma cells. Studies were done with normal epidermal melanocytes cell lines (HEMa-LP and NHEM-c cells) and malignant melanoma cell lines (A375 and SK-MEL-28). Exosomes were then isolated from the cell lines via ultrafiltration and ultracentrifugation. When an exosome pellet was finally produced, Nanodrop was used to determine the quantity. Transmission Electron Microscopy (TEM), RNA isolation and microarray analysis were then performed. TEM revealed that the vesicles were the correct size, between 50 – 100 nm and a western blot showed that CD81 was present in sample but not in cell lysates which confirms the vesicles were exosomes. mRNA genome arrays were conducted on all cell lines to distinguish different genes expressed in normal melanocytes versus cells with melanoma. Of the genes differentiated in the A375 cell line, many are implicated in N-glycan biosynthesis which is involved in melanoma cell metastasis.

The miRNA in exosomes has been shown to be strongly correlated with tumorigenesis and metastasis in the paper “Exosomes: proteomic insights and diagnostic potential” among many others. Thus, miRNA’s from the A375 cell line and the HEMa-LP cell line were looked at in order to differentiate. Around 230 miRNA’s were differentially expressed in A375 exosomes versus HEMa-LP exosomes. Many of these different miRNA’s, around 70, were associated with cancer. Furthermore, many other of these differentially expressed miRNA’s functioned in mechanisms that were closely tied with cancer, including, cell growth and proliferation, cellular development, cellular movement and cell death. Among those miRNA’s that were downregulated in the cancerous exosomes, were has-miR-31 and has-miR-185. These miRNAs are directly involved in the regulation of aggressive features of melanoma (Xiao, D. *et al.* 2012). The conclusion that can be inferred from these miRNA findings, is that exosomes that arise from melanoma contain many miRNA’s that are of great importance in the growth, metastasis and invasiveness of the cancer.

Protein profiles were produced on the A375 cell line and the HEMa-LP cell line to establish specific differences. The proteins annexin A1, annexin A2, syntenin-1, and hyaluronan and proteoglycan link protein 1 (HAPLN1), all have association with metastasis, invasiveness and angiogenesis. Annexin A1 and HAPLN1 were both upregulated in A375 exosomes while Annexin A2 and syntenin-1 were slightly downregulated. We can infer from these results that Annexin A2 and HAPLN1 have potentially more important and specific roles in the growth of the melanoma.

After a gene is transcribed into mRNA’s and miRNA’s they can then target various other genes that code for functional proteins. And as we have seen, exosomes from cancerous cells have differentiated genes, mRNA’s and miRNA’s and proteins. So, it is conclusive to

acknowledge that in this way, the genes, RNA's, and proteins that an exosome carries, incorporates all the essential messages, needed to continue the growth and metastasis of the tumor, to deliver to a none cancerous cell. The culmination of the work done in this lab was to prove just that. A375 exosomes were incubated with HEMa-LP cells and a migration/invasive assay was performed. It was showed that the percent invasion of these HEMa-LP cells after incubation were far greater than the control cells. This ultimately proves that exosomes from melanoma cells are mediators and play a significant role in metastatic growth.

Viral Exosomes

Virus's often use the host cells endocytic pathway as a means of entry and exit from the cell. This is primarily because the budding of viruses and the endocytic pathway both need membrane curving, packaging and budding for release. There are a number of different virus's that use clathrin-mediated endocytosis and enter the late endosomal pathway, fuse with ILV's and ultimately influence the biogenesis and contents of exosomes.

Biogenesis

A major way that virus's complete their life cycle and spread, is by hijacking the biogenesis of exosomes. They due this primarily by altering the ESCRT and Rab GTPase proteins which are essential in biogenesis of exosomes. The "Trojan Exosome hypothesis" states that HIV-1 uses the exosome biogenesis to package its capsid and uses the exosomes to further infection in the absence of viral envelope proteins (Fleming et al. 2014). Beyond this, the role of the ESCRT proteins in the development of the hepatitis virus family is well-studied. HCV (hepatitis c virus) has been shown to us the protein Hrs, part of the ESCRT-0 complex, to initiate the multivesicular bodies to uptake the viral capsid (Fleming et al., 2014). HAV and HEV

circulate in the body as membrane enclosed virions and avoid detection from the host immune response. In a recent paper it was suggested that the construction of these membrane enclosed virions involves the use of many integral ESCRT proteins (Khan et al., 2017). HAV requires CHMP2a which is part of the ESCRT-III complex, Alix, and VSP4 which is an ESCRT-I, and ESCRT-III associated protein (Khan et al., 2017). While HEV requires CHMP2a, Alix and Hrs which is part of the ESCRT-0 complex (Khan et al., 2017).

Other than the ESCRT pathways, the Rab GTPases are also utilized by many different viruses in order to continue their development. For example, in a study involving hantavirus-infected cells, interference with the Rab11 protein decreased virion production tenfold (Khan et al., 2017). This suggests that the hantavirus hijacks the Rab11 pathway and uses it to multiply and spread. Another Rab GTPase that is commonly hijacked is the protein Rab27a, that plays an important role in the fusion of exosomes with the plasma membrane. Viruses such as cytomegalovirus and HIV have been shown to upregulate the production and levels of Rab27a ultimately raising exosomal production (Khan et al., 2017).

Viral Markers

When analyzed, exosomes derived from virally infected cells yield major differences from exosomes that arise from uninfected cells. In a study done with infected B cells from the Kaposi's sarcoma herpes virus (KSHV), and/or the Epstein-Barr Virus (EBV), mass spectrometry profiled the various proteins that were present (Fleming et al., 2014). It was determined that there were 345 additional proteins incorporated in the infected cells exosomes than in the non-infected exosomes (Fleming et al. 2014). Different exosomes contained unique proteins that affected various bodily pathways. This insinuates that these hijacked exosomes have varying functions that may be complementary. A few of the pathways that these virus's

affected were metabolism, protein translation and cellular migration. However, protein content was not the only change in the viral exosome cargo. The mRNA, miRNA and small non-protein coding RNA cargo of exosomes released by virally infected cells was different to that of exosomes released by cells not infected by virus. Specifically, in the Epstein-Barr Virus, the virus' miRNAs paired with the natural miRNAs seem to help modulate the expression of target genes in the accepting cell (Fleming et al., 2014). These exosomes also have two small RNA's, EBER-1 and EBER-2, that are RNA polymerase II/III transcripts (Fleming et al., 2014). These RNA segments are untranslated and are implicated in oncogenesis. The exosomal biogenesis hijacking, combined with the release of viral contents in the exosomal cargo, shows how viruses can evade immunity, proliferate and increase infectivity.

A different way that virus's can use exosomes to further their reproduction was shown in a study on HIV. It has been shown that exosomes derived from cells infected by HIV can transmit the HIV virus receptors CCR5 and CXCR4, to non-infected cells, furthering the infection (Khan et al., 2017). Separately, viruses such as hepatitis C don't need receptors, and package their entire RNA genome within the exosomes (Khan et al., 2017). This is very beneficial for the virus because it reduces the chances of detection via antibodies

In a paper by Ahmed et al., it was shown that exosomes from EBV-infected B cells induce apoptosis in recipient cells, while non-infected B cells do not. In this same study, it was shown that the apoptosis was induced in the extrinsic pathway involving Fas-ligand (Fas-L), contained in the exosomes. While a different paper by Khan et al., showed that exosomes from lymphoblastoid cell lines contain the same ligand, Fas-L and Major Histocompatibility Complex II (MHCII) which induces apoptosis in CD4+ T cells. These two experiments together illustrate

how EBV can multiple and proliferate while suppressing the immune response, by inducing apoptosis in non-infected lymphocytes.

Bacterial Exosomes

When compared to cancerous and viral cells releasing exosomes, less is known about exosomes that arise from cells infected by a bacterium. In contrast to much evidence that viral exosomes help to spread and benefit the virus, exosomes from cells infected by bacteria have been shown to promote inflammation and other immune responses such as B and T cell stimulation and macrophage chemotaxis (Fleming et al., 2014). In other studies, infected exosomes effect on cytokines is more convoluted as there is evidence that the exosomes help cytokines induce B cell stimulation while also inhibiting T cell response. However, when looking at the effect of *Mycobacterium tuberculosis*, it has been concluded through a study by Singh et al., that exosomes from infected cells contain factors that down regulate immune genes such as nitric oxide synthase, PGE2 synthase, and HSP70. It can be reasonably inferred from this mixture of data that the role exosomes play in cellular communication during bacterial infections is multifaceted and complex and can differ tremendously according to different bacterial infections.

A study by Dr. Adam Fleming investigated the miRNA content of exosomes arising from THP 1 cells infected with either *Yersinia pestis* or *Bacillus anthracis*. It was concluded that the relative amounts of host miRNA were different in the infected cells as compared to the noninfected cells. Moreover, the differing type of infection seems to play a role in the exosomal packaging specificity. The figure below shows the changes in the amount of miRNA found in exosomes derived from cells infected with Y. Pestis or Bacillus anthracis. Each column signals

an individual exosomal miRNA, the red color denotes an increase in miRNA in exosomes in infected cells compared to control cells, while a green color denotes a decrease.

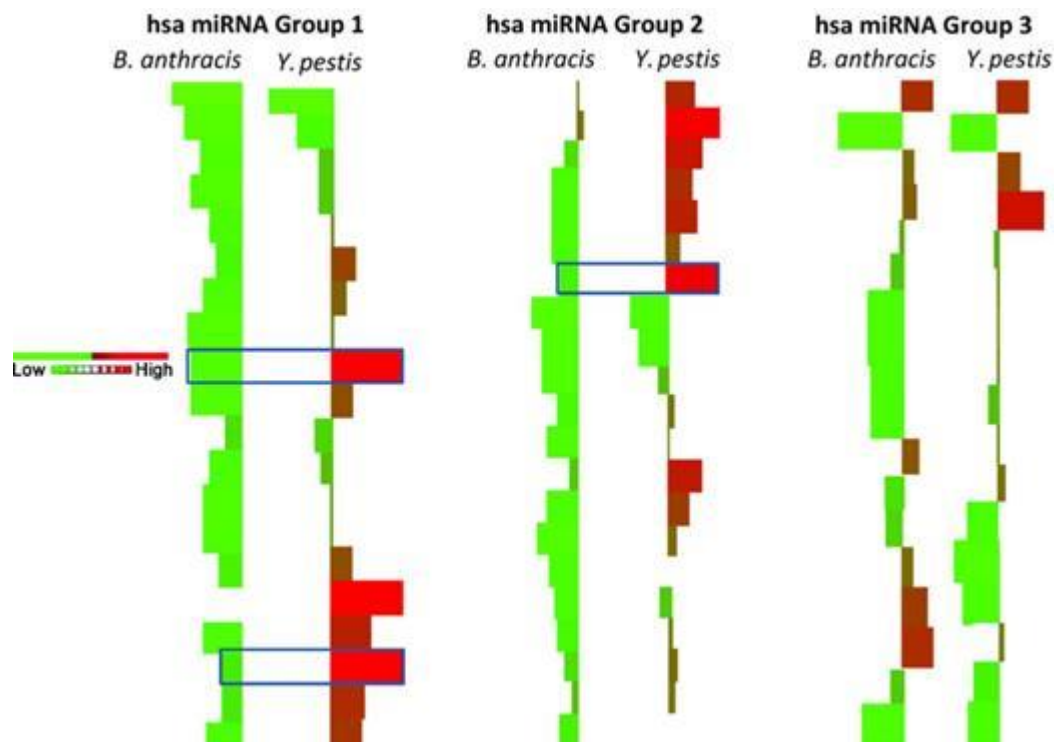


Figure 2: Variances in amount of exosomal miRNA due to differing bacterial infection. Red color signifies miRNA increase in exosomes derived from infected cells relative to the uninfected control, while green color signifies miRNA decrease.

Further studies have been produced to analyze proteomic constituents in exosomes arising from bacterially infected cells. One such study by Oehmcke et al., 2013 profiles the proteins in exosomes from cells that have been infected by *Streptococcus pyogenes*. The goal of this study was to do a proteomic profile of pro coagulant exosomes that result from cells infected by *S. pyogenes*. Exosomes were isolated and analyzed by mass spectrometry to identify proteins that were upregulated when exposed to M1. M1 is a virulence factor closely associated with *S. pyogenes*. It is thought that this protein will stimulate coagulation cascade pathways. The

results of the experiment yielded 169 proteins in the exosomes in both the control and the stimulated exosomes. The concentration of specific types of proteins was different however. The control group had the following concentrations: 57% of the proteins were cytosolic, 23% secreted, 12% membrane-associated, and 8% mitochondrial origin. While the cells stimulated with M1 released exosomes with: 35% of the proteins were cytosolic, 36% secreted, 28% membrane-associated, and 1% mitochondrial origin. Fibrinogen-binding integrin CD18 was also upregulated by 42 times and CD11b upregulated 7.8 times. lysozyme and neutrophil defensin 1, which are proteins that function as antimicrobials, were upregulated 3.7 and 2.8 times respectively.

The same paper revealed a study looking at the effects when pro-coagulant exosomes were applied to the local site of *Streptococcus pyogenes* infection in mice. Three groups of mice were infected with *Streptococcus pyogenes* and were either not treated, treated with control exosomes, or treated with pro-coagulant exosomes. The group treated with pro-coagulant exosomes had prolonged survival and significantly reduced mortality rates. This data further shows that these pro-coagulant exosomes are part of the body's innate defense and are part of an early immune response.

Exosomes in Therapy

The information currently being gathered within the scientific community regarding exosomes is changing our understanding of disease progression and potentially opening new doors and tools to help fight that progression. Knowing now how widely the contents of exosomes can vary. Having knowledge of the various contents, yielding insight into which cells are releasing which exosomes, will allow exosomes to be used as biomarkers for the diagnosis of different diseases. This has already been done in some cancer cases and could be a leap forward

in early detection of malignancies. A great example of this potential effectiveness was a study done on ovarian cancer.

Currently, the best biomarker available for detecting ovarian cancer is cancer antigen 125 (CA-125) (Salomon et al., 2015). However, there are major problems with CA-125. It can only legally be used in patients that already have been diagnosed with ovarian cancer and it meant to be used simply to monitor the disease. Also, it is only elevated in 50-60% of early stage cancer patients (Salomon et al., 2015). Exosomes on the other hand, have many characteristics that make them an ideal diagnostic tool, many of which have already been discussed. Exosomes are released from cancer cells and their cargo represents what cell type they come from and their environment. But exosomes have even more properties that further its potential as diagnostic tools. Exosomes can be collected from a several different biofluids including blood, urine or saliva. Once collected, the exosomes are highly stable, and can be easily separated from other common proteins found in the fluid. A couple proteins that have been identified as markers in ovarian cancer are CD24 and EpCAM (Salomon et al., 2015). Furthermore, when a single diagnostic marker is unavailable, exosomes may still help by displaying mutated DNA or RNA or oncogenic protein.

Biomarkers are an important part of the future for exosomes but are not the whole picture. In 2005 a study was performed by Dr. Michael A. Morse to test the effectiveness of exosomes for treatment in non-small cell lung cancer (NSCLC). The study centers around exosomes released from dendritic cells called “dexosomes”. These dexosomes are thought to prime T cells and help eliminate tumors in some rodents. They do this by containing within them MHC class I and II molecules and several tetraspan proteins such as CD63 and CD81. The dexosomes act as a relay system in the immune response in the following way. After obtaining

the antigen, a dendrite will incorporate MHC-antigenic peptide complexes into its dexosomes. These dexosomes will then stimulate naïve dendritic cells in the lymph nodes, giving them the ability to stimulate CD4+ and CD8+ T cells.

In the study, 9 patients were selected to receive these dexosomes, that were treated with tumor associated antigen MAGE to illicit a T cell response, with either stage IV or III NSCLC. The results of the study show a somewhat mixed response. 3 of the 9 patients showed positive signs of delayed type hypersensitivity. Furthermore, the peptide-specific immune response to MAGE was analyzed using ELISPOT in 5 of 9 dosed patients with one of these patients showing increased T cell precursor levels. In 2/3 patients who had analyzable specimen, an increase in CD4+CD25+ T cells as a percentage of CD4+ T cells was observed following completion of dexosome therapy when compared with baseline values. As an aside, the dexosomes seemed to, unexpectedly, increase the natural killer cells activity in half of the patients. 2 of the 9 patients had disease progression at the beginning of the treatment. Both of these patients experienced clinical stability for some time before progression resumed. 4 of the 9 patients were stable as treatment began. 2 of the 4 remained stable for more than 12 months following treatment. One patient died unexpectedly while the other remained stable for 3 months before progression. These results show that treatment with exosomes is a viable option. That exosomes or dexosomes can be manufactured and safely administered to patients in hopes of treating specific cancers.

Conclusion

Exosomes are nano sized extracellular vesicles that arise from the late endosomal pathway. They are proving to be integral components of cellular communication and play a part in

pathogenesis and the immune systems reaction. Depending on the type of disease, there are many ways forward in the world of exosomes.

Cancerous exosomes appear to be a mixed bag of proteins and but seem to contain identifiable markers. When looking for a way to exploit exosomes in their role of cancer development, the first answer now seems to be to use them in a diagnostic way. It is to develop lists of protein and/or RNA signatures that are identifiable to one specific cancer. In this way, screening for multiple various cancers can be simplified and easily obtained. Beyond this, more research could be put into using exosomes or “dexosomes” to treat certain cancers. The initial studies seem to show that exosomes role in cancer therapy is possible and may have great potential.

Going forward, exosomes in viral infections may be a good target for therapy and treatment. Specific proteins, such as Rab27a and Rab11 can be targeted for inhibition, as it has been shown that these proteins are vital in exosomal release and this pathway is regularly taken advantage of by viruses. Viruses seem to be the pathogen that most utilized exosomes and its pathway through the cell. In this way, the more we understand about exosomes, the more we will know about how viruses manage to spread in such a rapid manner.

As far as bacterial infections are concerned, procoagulant exosomes effect on mice infected with the bacteria *Streptococcus pyogenes*, was positive with an increased likelihood of survival. This demonstrates, although more testing needs to be done, that procoagulant exosomes should be considered as a future route in treating bacterial infections. In all three cases, more research and a better understanding may lead to many therapeutic benefits.

Cellular communication is a topic of intense study and interest. It is a vital step in pathogenesis and in the body's defense and immune response to a pathogen. Exosomes function in this process is starting to become illuminated. More study needs to be done to better understand the implications of exosomes in viral and bacterial infections. But the information already present paints a picture that both benefits the pathogen and stimulates an immune response in many cases. This suggests that exosomes are a microcosm of their tissue and environment and can potentially be targeted for diagnosis and possibly treatment. The job they hold in cancer metastasis is similar, but more complicated. Cancer exosomes seem to be directly incorporated into the development and advance of cancers with very little redeeming qualities. Though the potential place of exosomes in both diagnosis and therapy for cancers seems to be an exciting and promising avenue.

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